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Angiotensin-(1–7)/Mas axis integrity is required for the expression of object recognition memory

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ABSTRACT

It has been shown that the brain has its own intrinsic renin–angiotensin system (RAS) and angiotensin-(1–7) (Ang-(1–7)) is particularly interesting, because it appears to counterbalance most of the Ang II effects. Ang-(1–7) exerts its biological function through activation of the G-protein-coupled receptor Mas. Interestingly, hippocampus is one of the regions with higher expression of Mas. However, the role of Ang-(1–7)/Mas axis in hippocampus-dependent memories is still poorly understood. Here we demonstrated that Mas ablation, as well as the blockade of Mas in the CA1-hippocampus, impaired object recognition memory (ORM). We also demonstrated that the blockade of Ang II receptors AT1, but not AT2, recovers ORM impairment of Mas-deficient mice. Considering that high concentrations of Ang-(1–7) may activate AT1 receptors, nonspecifically, we evaluate the levels of Ang-(1–7) and its main precursors Ang I and Ang II in the hippocampus of Mas-deficient mice. The Ang I and Ang II levels are unaltered in the whole hipocampus of MasKo. However, Ang-(1–7) concentration is increased in the whole hippocampus of MasKo mice, as well as in the CA1 area. Taken together, our findings suggest that the functionality of the Ang-(1–7)/Mas axis is essential for normal ORM processing.

1. Introduction

It is well established that renin–angiotensin system (RAS) is one of the major systems in the regulation of cardiovascular function and fluid homeostasis. Furthermore, it has been shown that the brain has its own intrinsic RAS with most of all its components expressed in the central nervous system (von Bohlen und Halbach & Albrecht, 2006). Interestingly, the constituents of RAS expression are not restricted to the brain areas involved in the control of cardiovascular functions. RAS components are also expressed in brain regions involved in the processing of cognitive functions, like hippocampus (Chappell, Brosnihan, Diz, & Ferrario, 1989; Sirett, Bray, & Hubbard, 1981) and amygdala (Krizanova, Kiss, Zacikova, & Jezova, 2001; Von Bohlen und Halbach, Walther, Bader, & Albrecht, 2000; Yang & Raizada, 1999).

The majority of investigations about RAS role on the central nervous system (CNS) focus on angiotensin II (Ang II), considered the most important angiotensin peptide. When administered centrally, Ang II can either improve (Braszko, 2002; Braszko & Wisniewski, 1988) or impair memory (Bonini et al., 2006; Kerr et al., 2005). Regarding synaptic plasticity, Ang II blocks long-term potentiation (LTP) in the hippocampus (Armstrong, Garcia, Ma, Quinones, & Wayner, 1996; Denny, Polan-Curtain, Wayner, & Armstrong, 1991) and amygdala (von Bohlen und Halbach & Albrecht, 1998). Moreover, Ang II reduces NMDA receptors signaling through AT2 (angiotensin II receptor type 2) receptors-mediated mechanisms (Jing, Grammatopoulos, Ferguson, Schelman, & Weyhenmeyer, 2004; Schelman, Kurth, Berdeaux, Norby, & Weyhenmeyer, 1997; Schelman et al., 2004).

Another angiotensin peptide broadly studied is the angiotensin IV (Ang IV), which is the biologically active (3–8) fragment of Ang II (De Bundel, Smolders, Vanderheyden, & Michotte, 2008). Acute intracerebroventricular (i.c.v.) administration of Ang IV or its analog Nle1-Ang IV improves the performance of rats in a range of learning and memory tasks including passive avoidance (Braszko & Wisniewski, 1988), object recognition (Braszko, 2004), spontaneous alternation (De Bundel et al., 2009) and Barnes maze (Lee et al., 2004). In addition, Ang IV induces facilitation of LTP in CA1 area of the hippocampus (Kramar et al., 2001).

The action of angiotensin peptides on the brain is not limited to Ang II and IV. Several other angiotensins are recognized as being

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biologically active. Angiotensin-(1–7) is particularly interesting, because it appears to counterbalance most of the Ang II effects (Daemen, Lombardi, Bosman, & Schwartz, 1991; Strawn, Ferrario, & Tallant, 1999; Tallant, Ferrario, & Gallagher, 2005). For example, while Ang II blocks LTP, Ang-(1–7) appears to enhance LTP in the hippocampus (Walther et al., 1998).

The biological relevance of Ang-(1–7) has been reinforced by the identification of its receptor, the G-protein-coupled receptor Mas (Santos et al., 2003). The physiological function of the Ang-(1–7)/Mas axis has been supported, at least in part, by studies using the Mas-deficient mice (MasKo). The MasKo, on the mixed 129/C57BL/6 genetic background, showed higher anxiety compared to the controls, but no alterations were found in the Morris Water Maze task (Walther et al., 1998).

To further explore the effect of Mas ablation on learning and memory, we use MasKo in the FVB/N background and chose four different memory behavioral tasks: step-down inhibitory avoidance task, Y-maze and two distinct protocols of the object recognition (novelty and the spatial location of the objects). The choice of tasks encompass a broad spectrum of memory related processes (fear-related memory, immediate working memory and declarative-like memory) while, at least to some extent, recruiting areas known to express strong labeling for Mas. The Mas receptors are expressed in several brain regions: abundant in hippocampus (Martin, Grant, & Hockfield, 1992); the olfactory tubercle (medial part), the piriform cortex, olfactory bulb, amygdala, anterodorsal thalamic nucleus, hypoglossal nucleus; nucleus of the solitary tract, caudal and rostral ventrolateral medulla, inferior olive, parvo and magnocellular portions of the paraventricular hypothalamic nucleus, supraoptic nucleus, and lateral preoptic area (Becker, Etelvino, Walther, Santos, & Campagnole-Santos, 2007); while a weak to moderate labeling was present all over the neocortex and especially in the frontal lobe (Bunnemann et al., 1990).

The distinct behavioral tasks chosen have been demonstrated to involve the hippocampus as part of the neural circuitry underling memory processing. The well established, hippocampal-dependent, one-trial, step-down inhibitory avoidance task (Bekinschtein et al., 2007; Bekinschtein et al., 2010; Bernabeu et al., 1995; Bevilaqua, Kerr, Medina, Izquierdo, & Cammarota, 2003; Cammarota et al., 1995; Cammarota et al., 2008; Izquierdo et al., 2006; Rossato et al., 2006) was used to access fear-related memory. The spontaneous alternation behavior was accessed through the Y-maze, which is considered a hippocampal-dependent immediate working memory task (Carroll et al., 2007; de Castro et al., 2009; Hughes, 2004; King & Arendash, 2002a, 2002b; Pych, Kim, & Gold, 2006; Rosario, Ramsden, & Pike, 2006). Finally, we use two distinct protocols of the object recognition task to verify the effect of Mas ablation on declarative-like memory (Dere, Huston, & De Souza Silva, 2005, 2007). The two protocols evaluate distinct components of the recognition memory, the novelty and the spatial location of the objects (Barker, Bird, Alexander, & Warburton, 2007; Dere et al., 2005). There are studies showing that these protocols may require the hippocampus and other cortical structures, specially the perihinal cortex, differently (Winters, Forwood, Cowell, Saksida, & Bussey, 2004), please see also (Dere et al., 2007). In summary, two ORM tasks were chosen in order to perform a more comprehensive evaluation both in terms of the memory components and the neural substrates involved.

Furthermore, we took advantage of the specific Mas antagonist, A-779 (Santos et al., 1994), to verify the effect of the blockade of Mas in the hippocampus on the consolidation of the object recognition memory (ORM).

Considering that RAS encompasses different biologically active peptides, with a common precursor, the genetic absence of one component could unbalance the already complex interactions between the various angiotensins (reviewed by von Bohlen und Halbach & Albrecht, 2006). In fact, Ang-(1–7), in spite of its low affinity to AT1 and AT2 receptors, can produce nonspecific activation of AngII receptors (Rowe, Saylor, Speth, & Absher, 1995). Hellner and coworkers (2005) demonstrated that Ang-(1–7)-induced suppression of hippocampal-LTP in MasKo mice is mediated by a non-specific action of Ang-(1–7) on AT1 receptors. Thus, in order to further clarify the neurochemical basis of the ORM deficit in MasKo mice, we decided not only to quantify the levels of Ang-(1–7) and its main precursors (Ang I and Ang II), but also to evaluate the involvement of Ang II receptors in the memory processes.

2. Materials and methods

2.1. Subjects

Eight to 10 week-old MasKo_FVB/N mice (Alenina, Xu, Rentzsch, Patkin, & Bader, 2008) and age-matching FVB/N mice were used in the study. Animals were housed in groups of three to five per cage in a temperature-controlled room (22 ± 2 °C) with a 12:12 light–dark cycles. Food and water were provided *ad libitum*. All experimental procedures were carried out in the light phase.

All experiments were conducted according to National Institutes of Health guidelines for animal care. The protocols used here were approved by the Animal Care and Use Committee at the Federal University of Minas Gerais, Brazil (167/2008). The investigators were blind to the genotype of the mice during all experiments procedures.

2.2. Behavioral assessment tests

In order to suggest that a given biochemical system plays a role in learning and memory using mutant animals, it is imperative to show that these animals do not have any behavioral impairment that could account for the cognitive deficit (or improvement) observed. Thus, besides the proper intrinsic controls to all cognitive tasks evaluated, additional three other behavioral assessment tests were conducted: (a) Wire hang test (motor strength impairment); (b) Sticky paper test (somatosensory and proprioceptive perception) and (c) Visual placing response (visual depth impairment).

The wire hang test seeks to evaluate motor strength deficits in rodent models of central nervous system disorders. The test is described in detail elsewhere (Sango et al., 1996). In summary, the mouse is placed on the top of a wire cage lid (22 cm × 22 cm) and then, once the investigator is sure to cause the animal to grip the wires, the lid is turned upside down. The latency until the mouse falls off the lid is quantified (up to a cutoff time of 60s). The sticky paper test evaluates possible somatosensory impairments. We used the protocol described by Metz and Schwab (2004). Self-adhesive backed labels (0.7 × 1.1 cm) were placed onto the ventral side of the distal hind limb. Immediately, the animal was put into an arena (50 × 50 × 50 cm) and the latency for the first reaction to the stimulus (e.g. paw lifting, sniffing, biting, or removal) was recorded. The visual placing response tests the integrity of the visual sensory system and visual depth impairments. The test consists of suspending the mouse by its tail while slowly lowering the animal towards a solid surface, without ever allowing direct contact of vibrissae to the surface (Fox, 1965; Metz & Schwab, 2004). Response is rated according to a scoring system: 0 indicates no observable placing response 1 represents a weak placing response and a score of 2 points reflects a clear placing reaction (the head raises and forelimbs extend towards the surface). For all behavioral evaluation tests conducted, the independent Student's t test was used to compare means between genotypes. Results are presented as mean ± SD.

2.3. Inhibitory avoidance

The apparatus was a Plexiglas box (21 × 22.5 × 22.5 cm) with a 10 cm^2 acrylic platform on the left end of a series of steel bars (diameter 0.4 cm), spaced 0.6 cm apart, designed for use in mice, that made up the floor of the box (Insight Equipamentos, Ribeirão Preto, Brazil). For training, animals were gently placed on the platform facing the left rear corner of the box. When they stepped down and placed their four paws on the grid, they received a 2 s, 0.3 mA, scrambled foot shock (Prado et al., 2006). There was no habituation to the box. Mice are animals with innate high exploratory behavior thus having a very short step-down latency during training.

Memory retention was evaluated in a non-reinforced test session carried out at 1.5 h and 24 h to analyze short- (STM) and long-term memory (LTM), respectively. Data were analyzed and passed in the normality test, thus were expressed as mean ± SEM and further analyzed by One-way ANOVA repeated measures following Tukey's multiple comparison test.

2.4. Y-maze

Immediate working memory performance was assessed by recording spontaneous alternation behavior (SAB) during a single session in the Y-maze (de Castro et al., 2009). Each mouse, new to the maze (made in white wood with 30 cm long by 6 cm wide by 20 cm high), was placed at the end of one arm and allowed to move freely through the maze during an 8 min session. The maze was positioned at the exact same location for all procedures. The series of arm entries was recorded visually. An arm choice was added only when both forepaws and hind paws fully entered the arm. The maze was cleaned between experiments with 70% alcohol to remove any residual odors. Alternation was defined if mice entered different arms three times in succession from the results of consecutive arm entering. The number of overlapping entrance sequences (e.g. ABC, BCA) was defined as the number of alternations. The percentage alternation was calculated according to the following formula: [total alternation/(total arms entered − 2)] ∗ 100. Therefore, the following hypothetical sequence of arms entered by a mice: A, C, A, B, C, A, C, B, A, C would yield an alternation score of 75% ([6 alternations/(10–2) arms entered] ∗ 100). Random selection of goal arms yields an alternation score of 50%. We also analyzed the total number of arm entries as an index of locomotor activity, although it is not direct evidence. To determine if alternation scores were significantly above the chance (50%), we used one-sample *t* test. To comparison between groups we use the independent Student's *t*-test.

2.5. Elevated plus maze

Elevated plus maze is a method for the assessment of unconditioned anxiety-like behavior in rats and mice (Lister, 1987). The apparatus consists of two open arms and two closed arms, crossed in the middle perpendicularly to each other, and a center area. The maze was raised 30 cm above the floor. Mice are allowed to move freely between the arms during 5 min. The number of entries into the open arms and the time spent in the open arms are used as indices of open space-induced anxiety in mice (Voikar, Polus, Vasar, & Rauvala, 2005). Subsequently, the percentage of visits, as well as the percentage of time spent in the open arms was calculated. To comparison between groups we use the independent Student's *t* test.

2.6. Object recognition

All animals were given a single 20 min habituation session in an empty white plastic cage (50 cm × 40 cm × 20 cm); which was equally illuminated and with no spatial/visual cues. Twenty-four hours later, in the training session (TR) of the novel object recognition task (NOR), animals were allowed to explore two copies of an identical object during 10 min. Memory retention was evaluated during the test session (TT) carried out 1.5 h, to evaluate STM and 24 h, to evaluate LTM. During test session, with duration of 10 min, one object was identical from TR and the second was an object never before explored by the animal (Capettini, Moraes, Prado, Prado, & Pereira, 2011).

At least 48 h after the LTM test in the NOR task, the same animals, except one MasKo mouse, were submitted to spatial object recognition (SOR) (Dere et al., 2005). During the TR session, animals were allowed to explore two identical objects (distinct from those used in the NOR) during 10 min. One hour later, during the TT, one object was shifted to a novel location within the cage.

All objects (available in duplicate) presented similar material and size, but distinct color and shape. The objects used had been selected from a large pool of objects on the criterion that mice would spend approximately equal amounts of time exploring each of them (data not shown). Between trials, box and objects were cleaned with 70% alcohol and air-dried. Exploration time was defined as sniffing or touching the object with the nose (Capettini et al., 2011).

A digital camera was mounted on the ceiling above the box and connected to a computer with a video-tracking system that objectively monitored and quantified animals' movements (ANY-maze – Stoelting, Inc., Wood Dale, IL). The results are expressed as recognition index, RI, (time exploring the new object/total time exploring objects). To analyze if animals spent significantly more than 50% of exploration time with the novel or new located object we use One-sample *t*-test. This initial analysis is important to demonstrate that the animals have better than chance performance otherwise, the task does not measure object recognition memory. The RI values above 0.5 mean that the animal recognized the new object or new located object. To compare between groups, we used the independent Student's *t*-test (Fig. 2); one-way ANOVA (Fig. 3) or two-way ANOVA (Fig. 4).

2.7. Intra-hippocampus drug injection protocol

FVB/N mice were bilaterally implanted, under deep ketamin (70 mg/kg) and xylazine (10 mg/kg) anesthesia, with 26-gauge guides aimed to the CA1 region of the hippocampus in accordance to coordinates (DV 1.0; AP 1.94; LL 1.6) taken from the Mouse Brain Atlas of Paxinos and Franklin (1997) (Fig. 3C). Animals were allowed to recover for 4 days and then were submitted to the novel object recognition task.

Immediately after training session on NOR, a 33-gauge cannula was tightly fitted into the implanted guide and linked by an acrylic tube (P10) to a Hamilton micro syringe. Infusions (0.5 μL/side) were carried out using an infusion pump (0.5 μL/min); the 33-gauge cannula was left in place for 60 additional seconds to minimize back flow.

The drug used, A-779 ([D-Ala7]-ANG-(1–7), is a specific antagonist of Mas (Santos et al., 1994) and was administered in two different concentrations: 100 pmol and 500 pmol (Becker, Santos, & Campagnole-Santos, 2005; Fontes, Martins Pinge, Naves, Campagnole-Santos, & Lopes, 1997; Zhou et al., 2010). After the injection, the same animal were submitted to STM and LTM tests, 1.5 and 24 h, respectively.

Cannula placement was verified postmortem as described previously (Pereira et al., 2005). Briefly, 2–4 h after the behavioral test,

0.5 μl of a 4% methylene-blue solution were infused as described above and the extension of the dye 30 min thereafter was taken as indicative of the presumable diffusion of the vehicle or drug previously given to each animal. Only data from animals with correct cannula implants were included in statistical analyze. Data were expressed and analyzed as explained in the object recognition section, except the groups were compared by One-way ANOVA and Bonferroni's multiple comparison test.

2.8. Peripheral drug injection protocol

Losartan (Sigma–Aldrich) 10 mg/kg was administered by intraperitoneal (i.p.) injection, 18 h and 1 h prior training (Raghavendra, Chopra, & Kulkarni, 2001). It is well accepted that this dose of losartan crosses the brain blood barrier and functionally blocks their targets in the brain (Polidori, Ciccocioppo, Pompei, Cirillo, & Massi, 1996; Tota et al., 2009; Wang, Tan, & Leenen, 2003).

PD123319 (Sigma–Aldrich) 1 mg/kg was administered by subcutaneous (s.c.) injection, 12 h and 1 h before training session (Macova, Pavel, & Saavedra, 2009).

We did not observe any difference regarding i.p. and s.c. administration in the saline group (data not shown), then our saline group is composed by both i.p. and s.c. saline injected mice.

Data were expressed as recognition index. After the one-sample t-test analysis, as explained in Section 2.4, we performed the two-way ANOVA with Bonferroni post-test. The interaction between factors (treatment X genotype) was further analyzed by one-way ANOVA or independent *t*-test.

2.9. Radioimmunoassay (RIA)

Hippocampus or only the CA1 area was harvested from each animal and the samples were withdrawn into two different sets of ice-cooled tubes, one containing 7.5% ethylenediaminetetraacidic acid, EDTA, for total protein determination and the other containing protease inhibitors cocktail (0.01 mmol/L p-hydroxymercury benzoate; 1.5 mmol/L o-phenanthroline; 0.01 mmol/L para-methyl sulphonil fluoride; 0.05 mmol/L Pepstatin A, and 10 mmol/L EDTA) for angiotensin peptide measurements (50 μL per 1 mL of sample). Samples were centrifuged at 2000 × g for 20 min at 4 °C, and the supernatant was stored at −20 °C. Peptides were extracted onto Bond-Elut phenylsilica cartridge (Varian, USA). The columns were pre-activated by sequential washes with 10 ml of 99.9% Acetonitrile/0.1% heptafluorobutyric acid (HFBA) and 10 ml of 0.1% HFBA. Sequential washes with 10 ml of 99.9% Acetonitrile/0.1% HFBA, 10 ml of 0.1% HFBA, 3 ml of 0.1% HFBA containing 0.1% BSA, 10 ml of 10% HFBA and 3 ml of 0.1% HFBA were used to activate the columns. After sample application, the columns were washed with 20 ml of 0.1% HFBA and 3 ml of 20% HFBA. The adsorbed peptides were eluted with 3 ml of 99.9% Acetonitrile/0.1% HFBA into polypropylene tubes rinsed with 0.1% BSA. After evaporation, Ang I, Ang II and Ang-(1–7) peptides levels were measured by radioimmunoassay (RIA) (Botelho, Block, Khosla, & Santos, 1994). Proteins were quantified by the Bradford method (Bradford, 1976). Student's *t*-test was used to analyze the data.

3. Results

3.1. MasKo mice behave like FVB/N in the inhibitory avoidance, Y-maze, and elevated plus maze tasks

To date, the knowledge about the MasKo mice performance in hippocampus-dependent tasks is limited to the water maze (Walther et al., 1998). Considering that Mas is highly expressed in the hippocampus, we decided to determine if Mas ablation alters the performance of mice in distinct hippocampus-dependent tasks. To address this question, we evaluated FVB/N and MasKo mice, backcrossed to the same genetic background (Alenina et al., 2008), in the inhibitory avoidance (IA) and Y-maze tasks. We also evaluated the anxiety-like behavior in the MasKo, since it was demonstrated that on the mixed 129/C57BL/6 genetic background, MasKo present higher anxiety compared to the controls (Walther et al., 1998).

As shown in Fig. 1A, FVB/N ($n = 12$; $F(2,22) = 8.54$; $P = 0.001$) as well as MasKo ($n = 17$; $F(2,32) = 43.85$; $P < 0.0001$) are able to associate the platform step-down with the shock delivery, which is demonstrated by the increase in the latency in both STM and LTM tests, comparing to the training session. Furthermore, we did not observe difference between genotypes regarding training ($t(27) = 0.59$, $P = 0.55$), STM ($t(27) = 1.45$, $P = 0.15$) or LTM ($t(27) = 1.69$, $P = 0.10$) latencies.

We also analyzed the spontaneous alternation behavior (SAB) using the Y-maze. The above-chance levels of SAB reflect both a

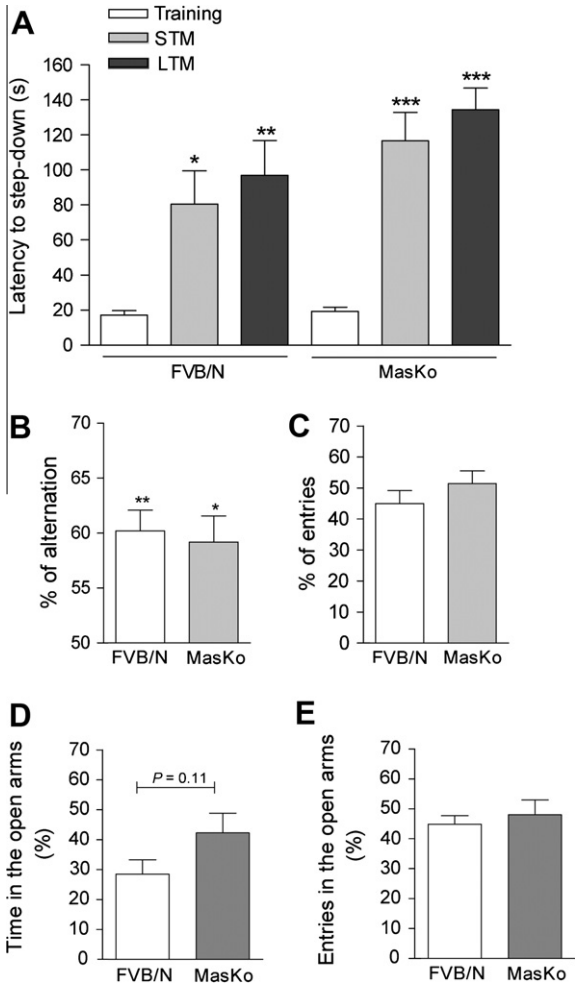

Fig. 1. MasKo mice exhibit normal inhibitory avoidance task (IA) memory, and spontaneous alternation behavior (SAB) in the Y-maze, as well as behave like control mice in the plus maze task. (A) Training, short- (STM) and long-term memory (LTM) latencies for IA task. MasKo ($n = 12$) and FVB/N ($n = 17$) exhibit significant increased latency in both STM and LTM tests comparing to the training session. Values are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$ comparing to the training session in the same genotype. (B) SAB in the Y-maze. FVB/N ($n = 20$) and MasKo ($n = 19$) exhibit significant alternation above the chance (50%) and (C) there was no difference between groups in the percentage of arms entered in the Y-maze. There was no difference between genotype ($n = 8$) in both (D) time and entries (E) in the open arms of the elevated plus maze. Values are mean ± SEM. $^{*}P < 0.001$; $^{**}P < 0.0001$ comparing to 50%.

predisposition to avoid recently visited locations, as well as intact working memory (reviewed by Hughes, 2004). As shown in Fig. 1B, both FVB/N ($n = 20$; $t(19) = 5.38$, $P < 0.001$) and MasKo ($n = 19$, $t(18) = 3.88$, $P = 0.001$) were able to alternate above the chance (50%) and there was no difference between groups in the number of arms entered ($t(37) = 1.1$, $P = 0.278$) (Fig. 1C). Our results demonstrated that Mas ablation has no effect on both IA and SAB.

Differentially to the MasKo on the mixed 129/C57BL/6 genetic background, the MasKo on the FVB/N background did not present anxiety-related behavior. As showed in the Fig. 1D and E, there was no difference between genotypes regarding the percentage of time ($t(14) = 1.69$, $P = 0.11$) or percentage of entries in the open arms ($t(14) = 0.55$, $P = 0.58$).

3.2. MasKo mice show no evidence of behavioral impairment

The behavioral assessment tests (wire hang, sticky paper and visual placing response) showed no evidence of behavioral impairment in MasKo mice. Motor strength evaluation, wire hang test, did not differ among genotypes: FVB/N ($n = 5$) and MasKo ($n = 5$) had latencies of 52 ± 17 s and 56 ± 7 s, respectively ($t(8) = 0.54$, $P = 0.59$). The latency to the first reaction to the adhesive backed labels place under the mouse hind limb also showed no difference between FVB/N ($n = 6$, 144 ± 52 s) and MasKo ($n = 6$, 159 ± 40 s) animals ($t(10) = 0.53$, $P = 0.6$). In the visual placing response test, all animals raised their heads and extended forelimbs towards the surface (FVB/N, $n = 6$, and MasKo, $n = 6$, all presented a maximum score of 2).

3.3. MasKo mice have impaired novel and spatial object recognition memories

Novel object recognition (NOR) is a form of visual paired comparison task that can be used to examine the role of the hippocampus in memory storage (Clark, Zola, & Squire, 2000; Rossato et al., 2007).

In a 1.5 h retention test, performed to examine short-term memory (Fig. 2A, STM), FVB/N mice present recognition index (RI) above 0.5 which indicates intact NOR memory ($n = 10$; $t(9) = 3.53$, $P = 0.006$). However, MasKo mice were unable to recognize the new object during the STM test session ($n = 8$; $t(7) = 0.14$, $P = 0.888$). Furthermore, unpaired t-test revealed statistical difference between groups ($t(16) = 2.502$, $P = 0.023$).

Similar results were obtained in the 24-h retention test (Fig. 2A; LTM). FVB/N mice showed intact NOR ($n = 10$; $t(9) = 6.38$, $P = 0.0001$) while MasKo mice did not ($n = 8$; $t(7) = 1.05$, $P = 0.32$). Moreover, there was statistical difference between genotypes ($t(16) = 2.38$, $P = 0.02$). These results cannot be attributed to differences in the total object exploration time during the training session (data expressed as mean ± SD: FVB/N = 42.6 ± 15.5 s; MasKo = 50.8 ± 16.6 s).

A modification of the NOR paradigm, named spatial object recognition (SOR), allows measurement of the memory for spatial locations within a familiar arena, where objects have been initially explored (Dere et al., 2007). FVB/N mice recognized the object presented in a different position during the test session ($n = 10$; $t(9) = 3.054$, $P = 0.01$), while MasKo show deficit in this task ($n = 7$; $t(6) = 0.481$, $P = 0.64$) (Fig. 2B). The comparison between groups showed statistical difference in the SOR ($t(15) = 2.437$, $P = 0.02$), with no difference regarding total object exploration time during the training session (data expressed as mean ± SD: FVB/N = 43.24 ± 20.67 s; MasKo = 47.82 ± 19.22 s). Our results showed that both novel and spatial object recognition memories are impaired in MasKo mice.

3.4. Intra-hippocampus A-779 infusion impaired NOR

Our results demonstrated that not all hippocampus-dependent memories are impaired in MasKo mice. In fact, we showed that specifically object recognition memory is impaired in MasKo mice. Based on this, and the fact that hippocampus presents high level of Mas expression (Metzger et al., 1995; Young, O'Neill, Jessell & Wigler, 1988), we investigate if the expression of ORM could be affected by the Mas blockade, directly into the hippocampus. To address this question we administered, in FVB/N mice, A-779 immediately post-training directly into the CA1 area of the hippocampus. One-sample t-test analysis revealed that mice which received saline intra-hippocampus were able to recognized the new object during STM (Fig. 3A; $n = 9$; $t(8) = 2.455$, $P = 0.03$) and LTM test (Fig. 3B; $n = 9$; $t(8) = 3.744$, $P = 0.005$). The lower dose of intra-hippocampus A-779 infusion (0.5ug/side) impaired both STM (Fig. 3A; $n = 9$; $t(8) = 1.737$, $P = 0.12$) and LTM (Fig. 3B; $n = 9$; $t(8) = 1.07$, $P = 0.31$) as well as the higher dose (2.5 ug/side) impaired STM (Fig. 3A; $n = 9$; $t(8) = 0.757$, $P = 0.47$) and LTM (Fig. 3B; $n = 9$; $t(8) = 1.742$, $P = 0.11$). The one-way ANOVA revealed a trend to difference between groups in the STM analysis ($F(2,24) = 3.325$, $P = 0.053$). Otherwise, the analysis of LTM data confirmed the difference between saline and A-779 (2.5 ug/side) group ($F(2,24) = 5.362$, $P = 0.01$). Our pharmacological data support the idea that hippocampal Mas blockade or ablation impaired NOR in mice.

3.5. AT1, but not AT2 blockade, prevents MasKo memory deficit

Considering that Mas may influence the signal cascades activated by the Ang II/AT1 axis trough heterooligomerization with

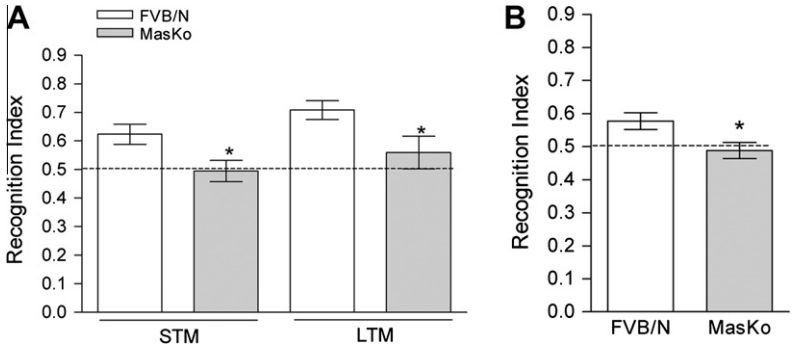

Fig. 2. Impaired object recognition memories in MasKo mice. (A) Recognition indexes (RI) for 1.5 h (STM) and 24 h (LTM) after training tests in the NOR task. FVB/N ($n = 10$) recognize the new object while MasKo ($n = 8$) did not. (B) RI for spatial object location task. FVB/N ($n = 10$) show significantly higher preference for the object located in a new place, but MasKo ($n = 7$) did not. Values are mean ± SEM. $^*P < 0.05$ indicates genotype effect.

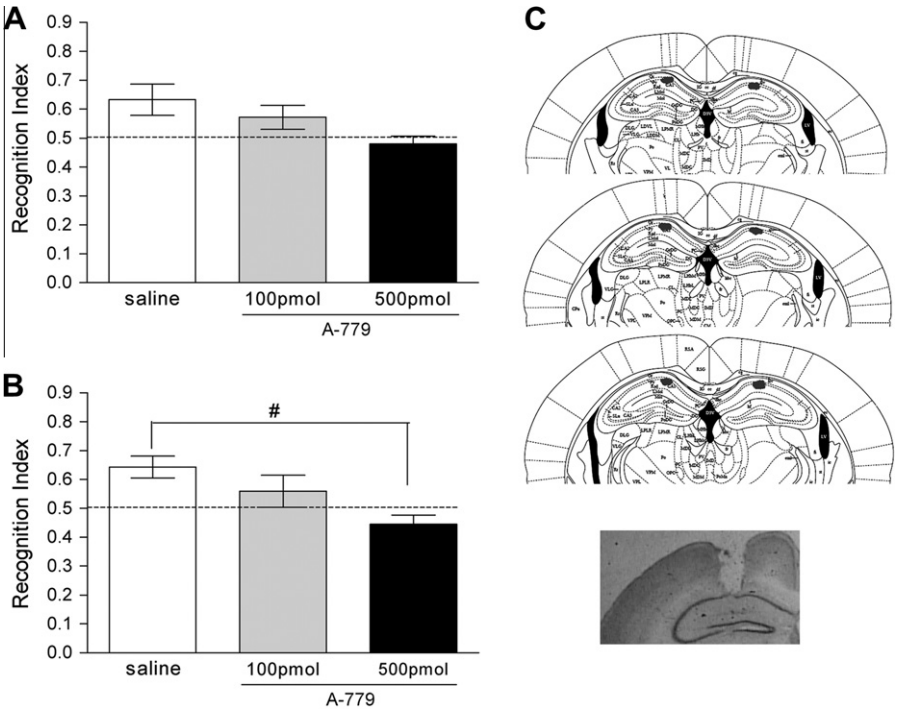

Fig. 3. Infusion of Mas antagonist, A-799, into the CA1 region of the dorsal hippocampus, blocks consolidation of NOR memory. Recognition indexes for STM (A) and (B) LTM in the NOR task. (C) Composite of infusions sites aimed at the CA1-hippocampus and photomicrograph of guide cannulae placement in dorsal CA1 of the hippocampus. Values are mean ± SEM. $^{\#}P < 0.01$.

AT1 receptor (Kostenis et al., 2005), we decided to evaluate the participation of the AT1, as well as the AT2 receptors, on the memory impairment observed in MasKo. To do so, we blocked these receptors by giving losartan and PD123319, specific antagonists of AT1 and AT2 receptors, respectively, before the initiation of the task.

Initially, we evaluated the inner group performance by comparing the recognition index with the value of 0.5 (one-sample *t*-test) to verify if there was a preference for one given object. The analysis of the STM test revealed that FVB/N mice (white bars, Fig. 4A) were able to recognize the new object after saline (SAL: $t(6) = 7.909$; $P = 0.0002$), losartan (LOS: $t(6) = 9.953$, $P < 0.0001$) or PD123319 (PD: $t(5) = 13.25$, $P < 0.0001$) administration. MasKo mice (gray bars, Fig. 4A) were unable to recognize the new object after saline (SAL: $t(6) = 0.366$, $P = 0.726$) or PD123319 (PD: $t(9) = 1.144$, $P = 0.282$), but their memory deficit was completed abolished by the pre-training administration of losartan (LOS: $t(7) = 13.14$, $P < 0.0001$). Since the two-way ANOVA revealed an interaction between the factors, genotype and treatment ($F(2,39) = 27.88$, $P < 0.0001$), we further analyzed the factors separately. The one-way ANOVA showed that both drugs diminished the recognition index of the FVB/N mice comparing to the saline group ($F(2,19) = 10.25$, $P = 0.001$). The comparison between treatments in the MasKo showed that LOS was different from the SAL and PD groups ($F(2,24) = 23.89$, $P < 0.0001$). We also found a statistical difference between genotypes in the saline ($t(12) = 4$, $P = 0.001$), losartan ($T(13) = 4.97$, $P = 0.003$) and PD ($t(14) = 2.17$, $P = 0.04$) groups.

The LTM analysis (Fig. 4B) revealed a similar pattern. All FVB/N groups present intact ORM (SAL: $t(6) = 5.418$; $P = 0.001$; LOS: $t(6) = 4.959$; $P = 0.002$; PD: $t(5) = 5.501$; $P = 0.002$). Again, MasKo present ORM deficit (SAL: $t(6) = 0.85$, $P = 0.42$) which was prevented by losartan (LOS: $t(7) = 4.949$; $P = 0.001$), but not by PD123319 administration (PD: $t(9) = 0.28$, $P = 0.785$). We found an interaction between the factors genotype and treatment

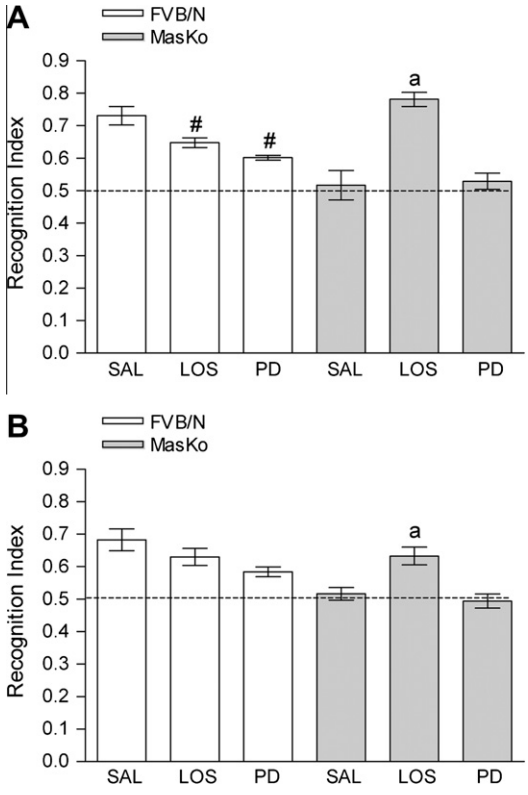

Fig. 4. The pre-training blockade of AT1 receptors prevents the NOR impairment expression in the MasKo mice. Recognition indexes of FVB/N and MasKo in the (A) STM and (B) LTM tests. Saline (SAL); losartan (LOS) and PD123319 (PD). Values are mean ± SEM. $^{\#}$Indicates difference from SAL group in the same genotype ($P < 0.001$) and a indicates difference from SAL group in the same genotype ($P < 0.05$).

($F(2,39) = 3.45$, $P < 0.05$), that we further analyzed separately. There was no difference between treatments in the FVB/N mice

($F(2,19) = 3.23$, $P = 0.06$), however the MasKo mice that received losartan were different from the group which received saline and PD ($F(2,24) = 6.59$, $P = 0.005$). There was a difference between genotypes in the saline ($t(12) = 2.27$, $P < 0.05$) and PD groups ($t(14) = 2.95$, $P = 0.01$), but not losartan groups ($t(13) = 0.08$, $P = 0.93$).

As we administered losartan and PD123319 before the task be initiated, the duration of object exploration during training brings an additional information regarding drug's effect on exploratory activity. In fact, PD123319 increases the total time of objects exploration in FVB/N [SAL = 31.1 ± 8.56 s; LOS = 29.39 ± 7.95 s; PD = 50.91 ± 19.15 s] ($F(2,25) = 7.36$, $p = 0.003$) and MasKo [SAL = 30.1 ± 13.37 s; LOS = 29.15 ± 8.41 s; PD = 55.75 ± 15.88 s] ($F(2,33) = 15.28$, $P < 0.0001$), comparing to saline and losartan ($P < 0.05$).

In conclusion, the results presented in Fig. 4 showed that the blockade of AT1 and AT2 receptors has distinct effects on ORM. In an animal with intact Ang-(1–7)/Mas axis, PD123319 and losartan diminished the performance, without affecting memory in general. However, if the Ang-(1–7)/Mas axis is altered by Mas ablation, losartan has a promnesic effect, while PD123319 has no effect on ORM of MasKo mice.

3.6. MasKo hippocampus presents high levels of Ang-(1–7)

We next investigated the consequences of Mas ablation on the brain renin–angiotensin system components. We choose to quantify, in the hippocampus, the principal activator of Mas, Ang-(1–7), and its main precursors, Ang I and Ang II, by RIA.

Our results demonstrated that there was no difference between genotypes regarding Ang I ($t(9) = 0.048$, $P = 0.96$) and Ang II ($t(7) = 0.06$, $P = 0.94$) concentrations. However, the concentration of Ang-(1–7) in the whole hippocampus from MasKo is higher in comparison to FVB/N mice ($t(8) = 2.81$, $P = 0.02$) (Fig. 5A). We also measured the Ang-(1–7) level in the CA1 area of the hippocampus. The results presented in the Fig. 5B reveal that MasKo mice have higher levels of Ang-(1–7) compared to FVB/N mice in the CA1 area ($t(6) = 2.3$, $P = 0.05$).

4. Discussion

It was demonstrated previously that MasKo mice, on the mixed 129/C57BL/6 genetic background, present higher anxiety compared to the controls, but no alterations were found in the Morris Water Maze task (Walther et al., 1998). Here we showed no differences between genotypes regarding anxiety and to further analyze the Ang-(1–7)/Mas axis modulation on memory, we chose three different hippocampus-dependent tasks and the object recognition was the only one affected by Mas ablation in the FVB/N genetic background. Furthermore, the post-training infusion of A-779, a specific antagonist of Mas, directly on CA1 area of the hippocampus of FVB/N mice, impaired the consolidation of object memory. Thus, both genetic ablation and pharmacological blockade of Mas impair ORM, showing clearly that the integrity of the Ang-(1–7)/Mas axis is required for the expression of this kind of episodic-like memory.

Although our behavioral assessment results show no evidence for motor strength, sensory perception or visual system impairment; caution must be taken before stating that there is no behavioral impairment in MasKo mice that could interfere with the interpretation of results from the memory tasks presented in this work. Nevertheless, pre-training data for each cognitive task evaluated, reinforces the hypothesis that there is no impediment of MasKo mice executing the task other than the cognitive aspect being tested. For example, in the Y-maze, both genotypes had similar spontaneous alternation behavior (Fig. 1B) and similar percentage of entrances in the maze's arms, suggesting proper exploratory behavior (Fig. 1C). If MasKo presented motor impairments, the exploration of the maze would most likely be compromised, but it was not. The same rational can be used for the plus maze evaluation tests. The mutants did not present either anxyolitic or anxiogenic behavior (Fig. 1D), and no difference between genotypes was found regarding entries in the open arms (Fig. 1E). Altogether these results suggest that the Mas ablation does not compromise exploratory activity in the mice. In addition, results from object recognition tasks also show no evidence of exploratory behavior impairments: since the time exploring the objects during the training session shows no statistical difference between MasKo and control FVB/N. In fact, the only observable deficit of MasKo mice in relation to controls was within the test session during a new object presentation task.

The declarative episodic memory provides us with data on what, when and where particular event occurred. In animals, we can evaluate a similar, but not necessarily equal, kind of declarative-like memory through the object recognition tasks (Dere et al., 2005, 2007). The consolidation of long-term object recognition memory requires hippocampal mRNA translation (Myskiw et al., 2008) and protein synthesis (Rossato et al., 2007), which in fact is similar to the inhibitory avoidance memory (IAM) consolidation (Cohen-Matsliah, Motanis, Rosenblum, & Barkai, 2010; Izquierdo, Cammarota, Medina, & Bevilaqua, 2004). However, ORM is formed based on the spontaneous tendency of animals to explore novelty and induces only low levels of emotional arousal (Roozendaal, Okuda, de Quervain, & McGaugh, 2006), while IAM is a classical emotionally arousing training task (Ferry, Roozendaal, & McGaugh, 1999; Introini-Collison, Miyazaki, & McGaugh, 1991). Thus, it seems that tasks with higher degree of emotional relevance are less sensitive to Ang-(1–7)/Mas axis modulation. This idea is partially supported by the fact that Ang-(1–7) infusion into CA1

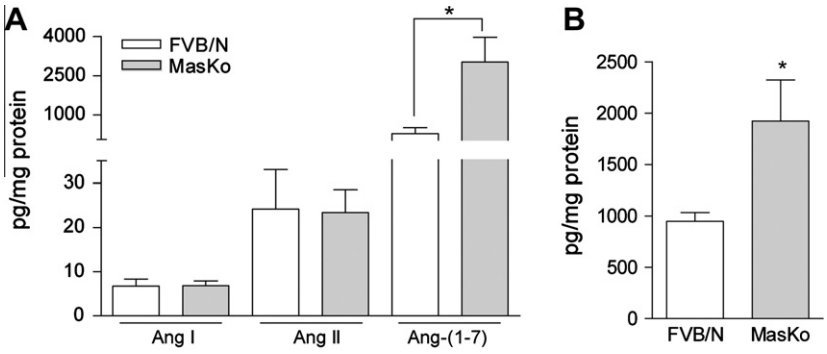

Fig. 5. Mas ablation increases the Ang-(1–7) levels in the whole hippocampus (A) and in the CA1 area (B). $n = 4–6$, $^*P < 0.001$.

region of the dorsal hippocampus does not affect retrieval of IA memory (Bonini et al., 2006). Our results demonstrated that Mas ablation compromises both STM and LTM in the novel object recognition task. As we submitted the same animal to the STM and latter to the LTM evaluation protocol, we have to consider the possibility that one test could be interfering with the other. Our results do not preclude an interference of the STM protocol in the consolidation/storage of the familiar object, presented twice, that might affect the behavioral outcome of the NOR LTM task. In blunt terms, there is no evidence in literature, that we know of, that conclusively dissociates the mechanisms underlying STM and LTM, in the NOR paradigm, if the same animal is used for evaluating both types of memory. However, taking in consideration that (1) we use distinct objects as the new element when evaluating both types of memory (i.e. one novel object presented during STM is different than the novel object presented during LTM evaluation) and (2) that the familiar object is identical for both tasks; one would expect a consolidation of the familiar object memory during STM evaluation and, consequently, an even better behavioral response during the LTM evaluation (i.e. with an even higher percentage of time spent in the novel object). This did not occur, which reinforces the LTM impairment interpretation of data, even more so if partially compensated by a possible consolidation during the STM protocol. Accordingly, the intra-hippocampal injection of A-779, immediately after training, statistically impaired LTM. Although differences were not statistically significant, the effect of A-779 in STM was similar to that observed in MasKo mice.

Concerning our experimental findings as to why MasKo mice are able to acquire and maintain the avoidance response associated with the IA task but not the declarative-like memory induced by the OR paradigm, our methodological approach does not directly address the specific mechanisms and differential involvement of the Ang-(1–7)/Mas axis in the neural substrates specific to either memory task. Nevertheless, it is possible to hypothesize on the reasons for this apparent discrepancy. The OR memory is based on the animal's spontaneous exploratory activity, with no obvious aversive component. Quite differently, IA memory relies on the association between an action (step-down the platform) and noxious stimulus (shock delivery in the paws). Thus, IA memory has to account for the innate interference in the behavioral response that is associated with the processing of noxious stimulation. Another hypothesis is that an overall hypersensitivity of MasKo mice to noxious stimulus could be compensating for memory deficits. It has been showed that Mas-related G-protein–coupled receptors (Mrgprs) are expressed on nociceptive, nonpeptidergic sensory neurons in the dorsal root ganglia (Dong, Han, Zylka, Simon, & Anderson, 2001; Lembo et al., 2002). Interestingly, a knockout mouse line, where 12 Mrgprs genes were deleted, showed prolonged mechanical and thermal pain hypersensitivity after hind paw inflammation (Guan et al., 2010). Based on this, it is possible that MasKo performance in the IA task is a result of an exacerbated associative response to the shock, rather than an intact fear-related memory. However, since we did not measure pain threshold in MasKo mice, this explanation is merely speculative, pending data from ongoing experiments.

Interestingly, PD123319 administration before training produced an increase in the object exploration time, independently of the genotype. Recently, it was demonstrated a positive correlation between the amount of object exploration during the training and the performance of the rats on the test session (Albasser, Davies, Futter, & Aggleton, 2009). Then, if the increase in the object exploration predicts memory enhancement, it should be expected a better performance of both genotypes under PD1234319 presence, but, in fact, we observed the opposite. The drug diminished the performance of FVB/N, without being amnesic and has no effect on MasKo mice memory, which is originally impaired. Thus, it could be assumed that the effect of PD123319 on object exploration time is unrelated to memory and probably independent of Ang-(1–7)/Mas axis functionality in the hippocampus. We also found that losartan and PD123319 diminished the performance of FVB/N mice on STM test, without affecting memory in general. As we administered both drugs peripherally, it is possible that other structures than the hippocampus are being affected by the drugs to generate the behavior effects.

We also demonstrated that the blockade of AT1, by losartan, rescue the memory deficit observed in MasKo mice. Importantly, this promnesic effect was exclusive for animals with Mas ablation and was not a consequence of differences in the object exploration time during the training. The classical Ang II effects on memory and synaptic plasticity through AT1 receptors activation are controversial. While some authors demonstrated a promnesic effect (Fogari et al., 2003; Tota et al., 2009), others show no effect (Bonini et al., 2006; Kerr et al., 2005) or even a negative modulation (Denny et al., 1991; Wayner, Polan-Curtain, & Armstrong, 1995). Indeed, the divergent signaling pathways used by AT1 receptor may have different roles in the array of behaviors induced by Ang II (Daniels, Yee, Faulconbridge, & Fluharty, 2005). Thus, divergent intracellular signals from a single receptor type can give rise to distinct behavioral phenomena. According, our results showed that losartan administration prior training did not alter the object exploration time in both genotypes, though decrease FVB/N performance in the NOR. However, the losartan effect on MasKo was opposite, it ameliorates the MasKo memory.

The fact that the memory deficits observed in MasKo mice are reversed with losartan strongly suggests that Ang-(1–7), also shown to have increased concentration in MasKo hippocampus, is probably exerting a deleterious effect through non-specific affinity to AT1 receptors. Nevertheless, such rational must be viewed with caution. The promensic effect on MasKo, especially considering that losartan was administered peripherally, could be due to Ang II tonic modulation on other areas. In physiological situations, Ang II activates AT1 to induce a cascade of intra-neuronal signaling events that ultimately leads to changes in membrane potential and an increase in neuronal firing (reviewed by Sumners, Fleegal, & Mingyan, 2002). The activation of neurons in cardiovascular control brain regions results in stimulation of the sympathetic nervous system, which mediates, at least partly, the cardiovascular complications associated with hypertension and heart failure (Carlson & Wyss, 2008; Liu, Gao, Roy, Cornish, & Zucker, 2006). Interestingly, MasKo in the FVB/N background exhibited higher blood pressures compared to the control (Alenina et al., 2008) with a possible autonomic balance shifted in favor of the sympathetic tone (reviewed by Alenina et al., 2008). In fact, the release of norepinephrine (NE) by the sympathetic nervous system plays an integral role in regulating processes involved in learning and memory (Holscher, 1999; Morilak et al., 2005; Roozendaal et al., 2006). In contrast, sustained exposure to NE can have detrimental effects on cognition (de Kloet, Oitzl, & Joels, 1999; McEwen & Sapolsky, 1995). Taken altogether, these results sprout an alternative idea regarding the positive effect of losartan on MasKo ORM task performance; which could be reducing the endogenously high sympathetic tone and, by doing so, allowing the memory expression of MasKo mice to normalize.

It has been demonstrated that Mas interacts with AT1 receptor and inhibit the actions of Ang II, thus being a physiological antagonist of AT1 receptor (Kostenis et al., 2005; Sampaio, Henrique de Castro, Santos, Schiffrin, & Touyz, 2007). Then, it is possible that in the absence of Mas, the antagonism exerted by this receptor is no longer active, which could be detrimental for NOR memory. Furthermore, we demonstrated that MasKo hippocampus present high levels of Ang-(1–7) and it is well known that Ang-(1–7) can activate AT1 receptors nonspecifically (Rowe et al., 1995). In this

scenery, the absence of the physiological antagonist and the high level of the Ang(1–7) could be converging to an exacerbate AT1 activation, with deleterious effects on ORM. In fact, Hellner and coworkers (2005) demonstrated that when the AT1 receptors were blocked by losartan in MasKo mice slices, Ang-(1–7) did not show its inhibitory effect on CA1-LTP. The authors suggested that Ang-(1–7)-induced suppression of LTP in MasKo mice was mediated by a non-specific action of Ang-(1–7) on AT1 receptors.

Our results strongly suggest an interaction between Mas and AT1 on the modulation of NOR memory, but are limited regarding the mechanism involved in this interaction. However, we can speculate that the crosstalk between both receptors could be happening, at least partially, via the modulation of the intracellular pathways dependent of the nitric oxide (NO) production, which is essential for NOR memory (Furini et al., 2010). In fact, Mas activation induces the release of NO (Sampaio et al., 2007; Dias-Peixoto et al., 2009; Feng et al., 2010) and, recently, it was demonstrated that Ang-(1–7) is capable of increasing *n*NOS-derived NO levels, in catecholaminergic neurons, through activation of Mas (Yang, Yin, Li, Zimmerman, & Schultz, 2011). Accordingly, MasKo exhibited impaired endothelial function, decrease NO production and lower endothelial NO synthase expression (Alenina et al., 2008).

In summary, the absence of the Ang-(1–7)/Mas axis effect on attenuate the actions of the AngII/AT1R pathway, through stimulation of nitric oxide release (reviewed by Xia and Lazartigues, 2008), is probably absent in the MasKo, which could be detrimental to memory.

5. Conclusions

The findings of the present study showed that the disruption of the Ang-(1–7)/Mas axis causes specific memory deficit in the object recognition task and increases Ang-(1–7) level in the whole hippocampus, as well as in the CA1 area. Furthermore, it is possible that the memory deficit observed in MasKo mice is due to AT1 receptors activation. These findings suggest, for the first time, Ang-(1–7)/Mas axis as an important modulator of object recognition memory.

Acknowledgments

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